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PAPER

Identification of roxarsone metabolites produced in the system: Soil–chlorinated water–light by using HPLC-ICP-MS/ESI-MS, HPLC-ESI-MS/MS and High Resolution Mass Spectrometry (ESI-TOF-MS)†

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Roxarsone (4-hydroxy-3-nitrophenylarsonic acid) in contact with soil of volcanic origin and chlorine containing water generated a set of organoarsenicals. The transformation products were identified with element-specific (ICP-MS) as well as molecular-specific (ESI-MS) detection after their HPLC separation. The identification of the main transformation products by means of ESI-MS, ESI-MS/MS and ESI-TOF-MS adduce evidence of chlorinated phenylarsonic acids and a phenylarsine oxide derivative which contains arsenic in the trivalent state. Traces of chlorine in water used for sorption experiments are suggested to be responsible for the formation of chlorinated products. After irradiation of a roxarsone solution with visible light, different transformation product so far not identified were detected.

Introduction

Phenylarsenicals play a key role in the environmental pollution caused by deposited chemical warfare agents[1] and animal medication.[2] Some phenylarsenicals, specifically, roxarsone (4-hydroxy-3-nitrophenylarsonic acid) and *p*-arsanilic acid (4-aminophenylarsonic acid), have been widely used for coccidiosis prevention and animal growth promotion in the poultry industry,[3–5] as chemotherapeuticals and also as effective growth promoters in the swine industry.[6,7] Roxarsone and *p*-arsanilic acid are bioaccumulated in very low concentration and are mainly excreted almost unchanged in manures.[5] In poultry litter the concentration of roxarsone ranges from 14 to 54 mg kg^{-1}.[8] In manures, also low concentrations of the metabolites: arsenate (As^{V}), arsenite (As^{III}), monomethylarsonic acid, dimethylarsinic acid, 3-amino-4-hydroxyphenylarsonic acid and 4-hydroxyphenylarsonic acid have been detected.[9]

Roxarsone is not naturally occurring in soils, but it is introduced into the soil through the use of poultry litter as fertilizer on cropland.[2,10] Garbarino *et al.* reported that roxarsone is stable in dried poultry litter, but when litter is in contact with water for certain incubation time; arsenate is the main degradation product.[11] In studies carried out with manure amended soil, arsenate was the main metabolite of roxarsone, as well.[9]

Recently we found that phenylarsenicals underwent sorption on volcanic soils from aqueous solution and unknown degradation products were formed after 24 h contact with the soil.[12] Due to the fact that roxarsone and its degradation products are highly water-soluble, they propagate into groundwater and can be accumulated by plants.[13] In this way, phenylarsenicals and their metabolites can be introduced into the food chain.[14–18]

In addition, phenylarsenicals can be subjected to transformation owing to microbial activity, increasing the toxicity and mobility of such degradation products. As an example, roxarsone can undergo biotransformation under anaerobic conditions to produce 3-amino-4-hydroxyphenylarsonic acid and inorganic arsenic species as main products.[19–21]

It is well known that the toxicity of arsenic highly depends on the chemical form in which it is present. It is generally accepted that the trivalent inorganic arsenic compounds are more toxic than the pentavalent one.[22] Also the methylated arsenic compounds are harmful and toxic to animals and humans.[16,23]

Due to the water solubility of the majority of the environmentally related arsenic species, high-performance liquid chromatography (HPLC) and capillary electrophoresis are the preferred separation techniques.[24,25] For arsenic speciation analysis, the most popular technique used so far, is HPLC coupled with mass spectrometry (MS). The combination of

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inductively coupled plasma (ICP) mass spectrometry for elemental identification and electrospray ionization (ESI) mass spectrometry for molecular identification as detector in HPLC is a powerful tool for the identification of unknown arsenic species.[24]

An increasing number of papers is devoted to the mobility and bioavailability of phenylarsenicals used in animal industry.[4,7,20,26] In one of the issues currently under discussion the behaviour of arsenic in cropland fertilized by poultry litter was investigated with respect to irrigation/rain and light exposition.[11,17,18]

The aim of our studies was to identify metabolites of organoarsenic compounds (especially derived from roxarsone) by suitable combinations of element-specific and molecular-specific mass spectrometric methods. The experiments were performed under simulated environmental conditions by interactions of roxarsone with volcanic soils, chlorinated water and under light irradiation.

Experimental

Materials and methods

Soil samples. The soils under investigation are widely distributed in Mexico and are constituents of the croplands there. All are from the type loamy clay soil and contain considerable amounts of iron oxides. The Mexican soils were derived from volcanic materials: *Acrisol* (AT) from Atécuaro in the state of Michoacán (19°34′60″ N, 101°10′60″ W), resulting from old volcanic ashes, *Tepetate* (TL) from Tlalpan in the state of Tlaxcala (19°16′60″ N 99°10′0″ W), corresponding to a volcanic tuff, both with a high level of degradation and fitted out for agricultural activities[27] and Andosol (LO) from La Loma in Amanalco de Becerra, state of México (19°15′ N, 100°01′ W). The fine fractions (<2 μm) of Acrisol and *Tepepate* are made up of two low activity clays: kaolinite in the Acrisol and tubular dehydrated 0.7-nm halloysite in the *Tepetate*. Akaganeite is the principal Fe-mineral component in the Acrisol with trace concentrations of goethite and hematite.[28] Andosol corresponds to redistributed volcanic ashes. The main minerals in its fine fraction (<2 μm) are allophane and Fe minerals, Fe–Al complexes and halloysite are present in a lower proportion additionally.[29]

Sorption, irradiation and chlorination experiments. Non-sterilized soils were used in the experiments. The soils were dried at 100 °C for 24 h, prior to use and afterwards sieved and homogenized. Each 3 mL of deionized water (DIW) containing roxarsone (740 mg L^{-1} as As) were added to each 100 mg of soil (size fraction < 0.71 mm) without any pH adjustment. The soil suspensions were shaken manually and kept afterwards in a water bath at 25 °C for 24 h under batch conditions. In the following the samples were filtered (RC membrane, 0.45 μm, Sartorius) and centrifuged for 30 min at 13,000 rpm. The supernatants were collected and stored in Eppendorf tubes at room temperature for subsequent analysis.

For the irradiation experiments a 702 UV-digester (Metrohm, Herisau, Switzerland) equipped with a high pressure mercury lamp HBO 500 was used. The light intensity of this lamp is 2,850 cd and the average light density amounts 30,000 cd cm^{-2}.

Table 1 Parameters for ICP-MS, ESI-MS, ESI-MS/MS and ESI-TOF-MS

ICP-MS (Agilent 7500ce)	**Conditions**
RF power	1500 W
Plasma gas flow rate	Ar 15 L min^{-1}
Carrier gas flow	0.5–0.7 L min^{-1}
Sampling depth	6–7 mm
Ion monitored	*m*/*z* 75 (As^+)
ESI-MS (Agilent MSD 6130)	
Polarity	negative
Capillary voltage	4000 V
Fragmentor voltage	200 V
Nebulizer pressure	276 kPa
Scan range	100–300 *m*/*z*
Spray temperature	350 °C
Nitrogen flow	11 L min^{-1}
ESI-MS/MS (API 2000)	
Polarity	negative
Capillary voltage	5000 V
Ion source	Turbolon spray
Collision energy	15 V
Product ion scans	molecular ions $[M - H]^-$ as precursors
Spray temperature	300 °C
ESI-TOF-MS (micrOTOF)	
Polarity	negative
Capillary voltage	4000 V
End plate offset	500 V
Capillary exit	150 V
Nebulizer gas	(N_2): 40 kPa
Drying gas	(N_2): 4.0 L min^{-1}
Drying temperature	200 °C
Skimmer	1: −50 V, 2: −23 V
Hexapole voltage	1: −23 V, RF: 100 V
Transfer time	49 μs
Pre-pulse storage	10 μs

Chlorination was performed by bubbling chlorine gas through an aqueous solution of roxarsone. The chlorine gas was produced by oxidation of hydrochloric acid with potassium permanganate.

Analytical methods

HPLC-ICP-MS/ESI-MS. For arsenic speciation, the samples were analyzed using an HPLC-ICP-MS/ESI-MS equipment consisting of μ-LC Series 1100 (Degasser, binary pump, thermostated autosampler) coupled with ICP-MS 7500ce and ESI-qMS 6130 in parallel (all Agilent Technologies, Santa Clara, USA) by splitting the mobile phase 1 : 1 by a T-piece. The injection volume used was 8 μL.

For identification of the phenylarsonic compounds the ICP-MS peaks at *m*/*z* 75 (As) were compared with those peaks obtained by the ESI-MS detector after their separation by means of reversed-phase chromatography (column: Atlantis dC18 (5 μm, 4.6 × 150 mm, Waters, Milford, MA, USA); eluent A: 0.1% HCOOH, 0.1% CH_3OH; eluent B: 0.1% HCOOH, 20% CH_3OH). The following eluent composition (gradient) was used: 0–3 min 100% A; 3–20 min 25% A (linear); 20–30 min 25% A; 30–31 min 100% A; 31–35 min 100% A. The conditions for the detection are listed in Table 1.

HR ESI-TOF-MS. For high-resolution mass spectrometry, a micrOTOF-MS system (Bruker Daltonics, Bremen, Germany)

in conjunction with a coaxial sheath-liquid ESI sprayer (Agilent Technologies, Waldbronn, Germany) with an incorporated fused silica capillary was used.

A mixture of propan-2-ol and water (50 : 50 v/v) containing 0.2% formic acid served as sheath liquid and was pumped with a flow rate of 3 μL min^{-1} using a syringe pump model KDS 601553 (KD Scientific, Holliston, MA, USA). Sample introduction was performed applying pressure to the sample vial which was connected with the coaxial sheath-liquid sprayer *via* a short piece of fused silica capillary (50 μm I.D., 360 μm O.D.). As a result, a sample flow through the capillary was generated. The parameters for the operation of the micrOTOF system are also summarized in Table 1.

HPLC-ESI-MS/MS. For HPLC-ESI-MS/MS measurements the following equipment was used:

HPLC: Agilent 1100 with autosampler (Agilent Technologies, Santa Clara, USA); ESI-MS/MS: Triple Quadrupole LC/MS/MS Mass Spectrometer API 2000 (Perkin-Elmer Sciex Instruments, Waltham, Massachusetts, USA). The parameters for detection are presented in Table 1.

Quality control

Blank samples were extracted and analyzed simultaneously with the soil containing samples to determine the potential lability at 25 °C of the roxarsone used in these studies. All experiments were repeated three times and each sample was also analyzed three times.

The total concentrations of arsenic in the resulting solutions were determined by ICP-atomic emission spectrometry (CIROS, Spectro A.I., Kleve, Germany).

Results and discussion

HPLC-ICP-MS/ESI-MS

For the separation of roxarsone and its transformation products reversed phase chromatography was chosen using gradient elution with methanol. The element-specific and molecular-specific detection was performed as described above by parallel coupling ICP-MS and ESI-MS. Overlaying the ICP-MS chromatogram with that of the ESI-MS facilitates the molecular mass search for arsenic containing compounds. In Fig. 1 the chromatograms of a freshly prepared roxarsone solution (ROX) in comparison to roxarsone treated 24 h by three different soils (AT, LO, TL) employing ICP-MS detection are presented. When roxarsone was in contact with the soils, additional peaks in the arsenic selective chromatograms could be detected indicating that besides sorption on the soil, also more than 10 transformation products were formed during the experiment. They were produced in different amounts as indicated by the peak heights/areas in the chromatograms detected by ICP-MS. The highest degree of transformation of roxarsone was observed using the volcanic soil TL. Because of the limited sensitivity of ESI-MS detection, the identification was focused on the major arsenic containing transformation products at retention times 6.3 (peak A), 16.2 (peak B) and 24.5 (peak C) minutes. They could be identified by their molecular mass peak in the scan - mode spectra using negative ionization (Fig. 2). Thereby peak A corresponds

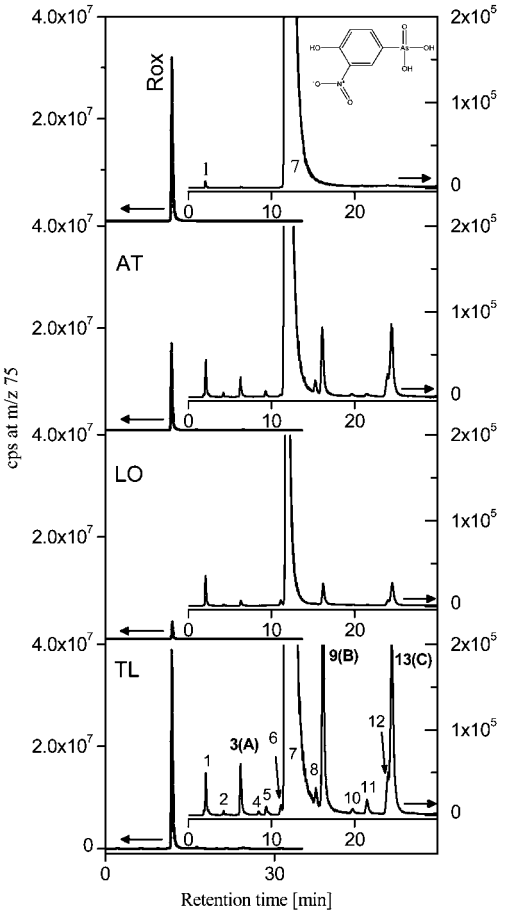

Fig. 1 HPLC-ICP-MS chromatograms of the initial solution of roxarsone (ROX) and solutions containing soluble transformation products detected after 24 h contact with the *Acrisol* (AT), *Andosol* (LO) and *Tepetate* (TL) soils: Left axis – full scale chromatograms; right axis – zoomed chromatograms.

to *m/z* 289, peak B to *m/z* 296 and peak C to *m/z* 280 (each as $[M - H]^-$). In the case of peaks B and C the isotopic pattern indicates the presence of chlorine in the molecule, which can be observed for the molecular mass peak as well as for the fragments.

HR ESI-TOF-MS

To determine the exact molecular masses of the unknown arsenic containing components, the solution carrying the transformation products of roxarsone was injected directly in an ESI-TOF-MS without chromatographic pre-separation. Fig. 3 shows the resulting mass spectra in the range of 275 to 300 *m/z*. The *m/z* – values in the high resolution mode agree with the results obtained by ESI-MS and ESI-MS/MS (Tab. 2). The differences (Δm) between the theoretical masses and the measured ones of the identified compounds A, B and C vary within 0.7 and 2.1, which is an acceptable value (< 3 ppm) for those measurements. Also the isotopic pattern of the sample compared with the simulated isotopic pattern (SIP) for peaks B and C, respectively, proved that these both compounds are chlorine-containing phenylarsonic acid derivatives as illustrated in Fig. 4.

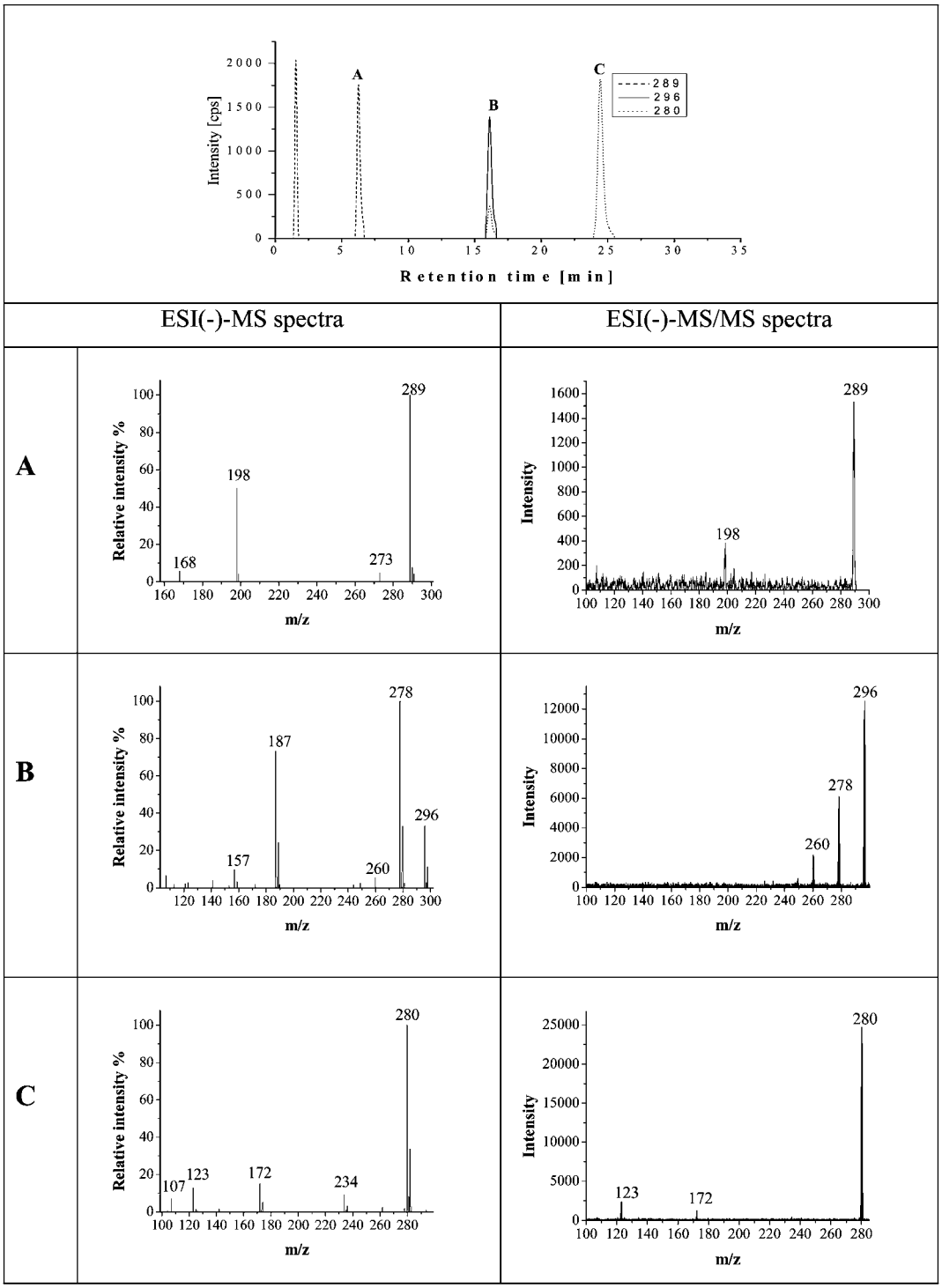

Fig. 2 HPLC-ESI-MS chromatograms in SIM mode on *m/z* 280, 289 and 296, and negative ESI-MS and ESI-MS/MS spectra of the peaks in the scan mode (*m/z* 100–300) of roxarsone solution in contact with the TL soil.

HPLC-ESI-MS/MS

For a positive proof of the structure of the transformation products the findings by HPLC-ICP-MS/ESI-MS and HR ESI-TOF-MS have to be supported by HPLC-ESI-MS/MS studies. These investigations were performed in the negative ionization mode. The precursor ions $[M - H]^- = 280$, $[M - H]^- = 289$ and $[M - H]^- = 296$ were analyzed by product ion scans. In general, the resulting product ions generated by collision activated dissociation (CAD) showed a good conformity with those one detected in the spectra using the scan mode ESI(-)-MS shown in Fig. 2.

For peak A, the precursor ion at *m/z* 289 was fragmented in one main product ion at *m/z* 198 (A2). The other fragments at

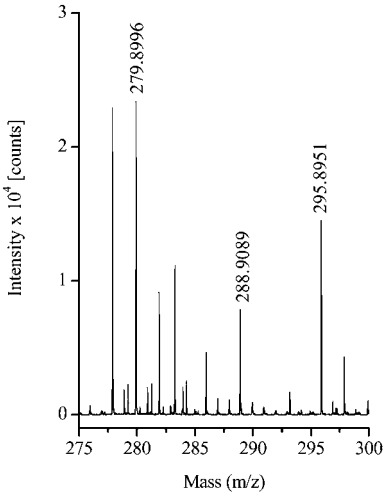

Fig. 3 ESI(-)-TOF-MS spectrum of the roxarsone solution after contact with the *Tepetate* soil.

m/z 273 (A4) and 168 (A3) detected by ESI(-)-MS could not be confirmed by ESI-MS/MS because of its lower sensitivity. For peak B, the precursor ion at *m/z* 296 was fragmented in two main product ions at *m/z* 278 and 260. The first fragment was also observed in the ESI(-)-MS studies with high intensity. The second one shows only a low abundance in the ESI(-)-MS spectra without an isotopic pattern of chlorine. The precursor ion at *m/z* 280 (peak C) was fragmented in two small product ions corresponding to *m/z* 172 and 123 corresponding to AsO_3^- shown in spectrum C in Fig. 2. It is not to be excluded that a co-eluting compound can generate this fragment. However, the simultaneous occurrence of *m/z* 107 for AsO_2^- and 123 for AsO_3^- additional to *m/z* 75 obtained by ICP-MS detection can be regarded as indication for the real existence of this fragments. The fragment *m/z* 234 was only detected by ESI(-)-MS as a chlorine containing one.

Proposals for the structure of the transformation products on the basis of the molecular masses and their fragmentation schemes. With the results obtained by the four mass spectrometric techniques applied in this study the fragmentation schemes for peaks A, B and C presented in Fig. 5 are suggested. For peak A, two isomeric structures of the arsenic-containing compound can be taken into account: 4-hydroxy-3,5-dinitrophenylarsine dioxide (R-AsO_2) or alternatively 4-hydroxy-3,5-dinitrophenyl arsenite based on the molecular mass at *m/z* 289 (4-(arsoryloxy)-2,6-dinitrophenolate, A1). First, fragments at *m/z* 273 (A4) and at *m/z* 198 (A2) confirm the existence of the dinitrophenyl structure of transformation product A1. Second, the fragment at *m/z* 198

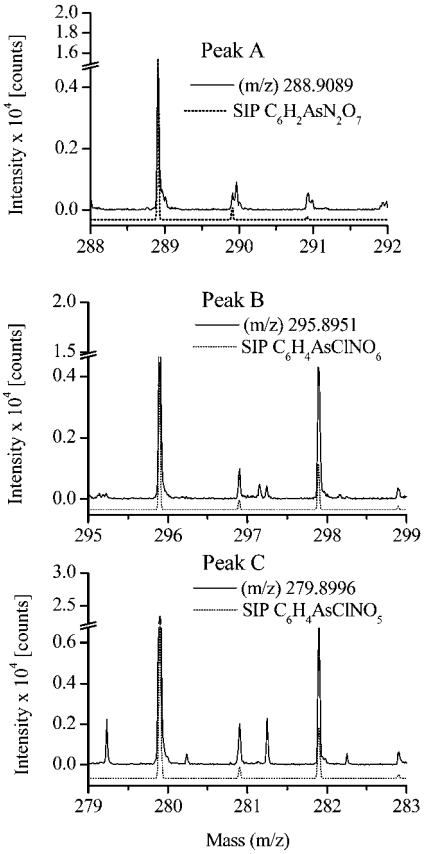

Fig. 4 Comparison of the ESI(-)-TOF-MS spectra with simulated isotopic pattern (SIP).

can be formed by the loss of AsO to form the radical 2,6-dinitro-4-oxocyclohexa-2,5-dienolate fragment (A2). A phenylarsine oxide like derivative (A4) was formed by the loss of oxygen from A1. A denitrification of A2 can lead to fragment A3 (*m/z* 168) a 5-nitro-3,6-dioxocyclohexa-1,4-dienolate.

The compound indicated as peak B is proposed to be the anion of 3-chloro-4-hydroxy-5-nitrophenylarsonate based on the molecular mass at *m/z* 296 (B1). By loss of water (Δ *m/z* 18) the main fragment 3-chloro-4-hydroxy-5-nitrophenylarsenite with *m/z* 278 (B2) was formed that is further fragmented to the radical anion 2-chloro-6-nitro-4-oxocyclohexa-2,5-dienolate fragment with *m/z* 187 (B3, Δ *m/z* 91) by cleavage of AsO. The elimination of oxygen results in 2-chloro-6-nitrophenolate *m/z* 157 (B4, Δ *m/z* 15). Alternatively, the fragment 4-hydroxy-5-nitrophenylarsenite (B5 *m/z* 260) derived from B2 was formed by substitution of chlorine by a hydroxyl group on the aromatic ring.

Table 2 Comparison of the molecular masses obtained with ESI(-)-MS, ESI(-)-MS/MS and ESI(-)-TOF-MS

		MS		MS/MS		TOF-MS		
Peak	Formula	Mass	Fragments	Precursor ion	Product ions	Mass	Theoretical mass	Δm (ppm)
A	$C_6H_2AsN_2O_7$	288.8	See Fig. 2 and 5	289	198.3	288.9089	288.9083	2.1
B	$C_6H_4AsClNO_6$	295.8		296	278.3; 260.0	295.8951	295.8949	0.7
C	$C_6H_4AsClNO_5$	279.8		280	172.3; 123.0	279.8996	279.8999	1.1

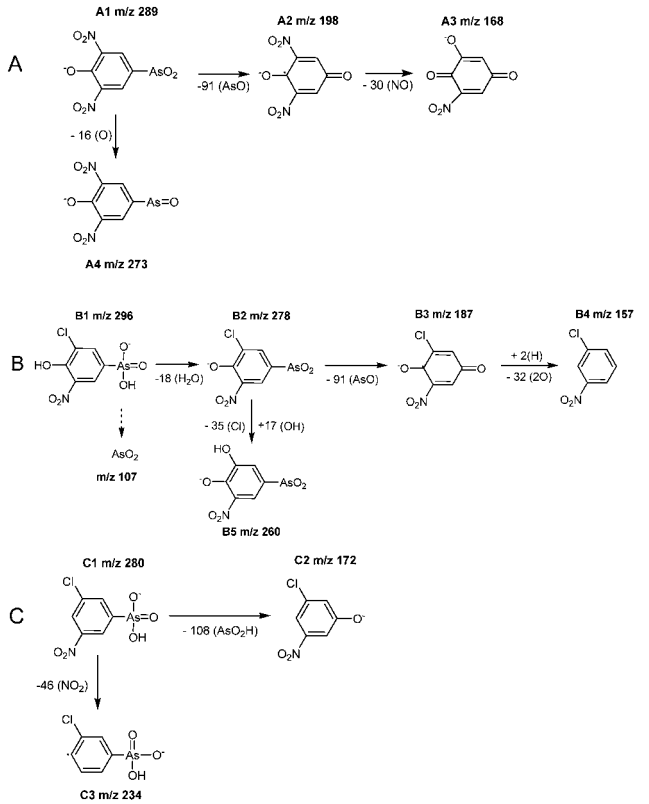

Fig. 5 Schemes proposed for fragmentation pathways to identify the peaks A, B and C in Fig. 2.

In the case of peak C, the proposed compound is 3-chloro-5-nitrophenylarsonic acid based on the molecular mass at *m*/*z* 280 (3-chloro-5-nitrophenylarsonate, C1). The loss of the arsonate group (AsO_2H) results in 3-chloro-5-nitrophenolate at *m*/*z* 172 (C2, Δ *m*/*z* 108). Considering an alternative pathway the anionic radical fragment 3-chlorophenylarsonate at *m*/*z* 234 (C3, Δ *m*/*z* 46) was detected, which can be attributed to the lost of the nitro group.

Preliminary investigations for mechanistic examinations. In order to understand the formation of transformation products of roxarsone which contained As(III) (Fig. 5 A1) as well as chlorine as substituent (Fig. 5 B1 and C1) several preliminary experiments were carried out. Initial point of the investigations was to adsorb roxarsone on different soils for its elimination (Fig. 1, full scale view). Therefore, roxarsone solutions prepared with deionized water were agitated with soils. How one can see in Fig. 1 the transformation of roxarsone (reduction and electrophilic substitution) occurred within some hours during this 'simple' process. The chlorination could be attributed to relatively high chlorine content in Mexican tap water used to prepare deionized water. Dissolved chlorine gas cannot be retarded on the ion exchange resin and activated carbon applied for DIW preparation. The pretreated tap water contains chlorine in a concentration range of 10 to 20 mg L^{-1} and the corresponding DIW < 10 mg L^{-1}. To ensure this assumption several preliminary experiments were performed. The investigations were focused on the influence of dissolved

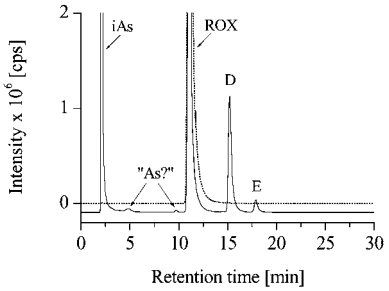

Fig. 6 HPLC-ICP-MS chromatograms of untreated (dotted line) and chlorine-treated (solid line) roxarsone solutions; (iAs) inorganic arsenic.

chlorine on chlorination of roxarsone. Furthermore, irradiation with UV and visible light was also applied to initiate transformation reactions on roxarsone.

Influence of chlorine on roxarsone transformation. In a first trial, chlorination of the aromatic ring of roxarsone was performed by bubbling chlorine gas through a roxarsone solution. The resulted chlorine concentration was around 500 mg L^{-1} which should facilitate (i) the faster formation of chlorinated roxarsone products and (ii) should lead to higher product yields. Fig. 6 shows the HPLC-ESI-MS/ICP-MS chromatogram of the aqueous solution of roxarsone before and after treatment with chlorine for 10 min. Compared with the chromatograms shown in Fig. 1, two additional roxarsone derivatives were formed in the retention time window of 14 to 19 min. The retention time of peak D in Fig. 6 corresponds well with the retention time of peak B (3-chloro-4-hydroxy-5-nitrophenylarsonate) in Fig. 1. The *m*/*z* value of the peak corresponds to the arsenic containing compound B1 (Fig. 5). A second peak E with a retention time of 17.9 min (Fig. 6) was not consistent with one of the peaks in Fig. 1.

After longer treatment, roxarsone was degraded completely, and a couple of new arsenic containing compounds in the retention time window of 2 to 5 min appeared. Further experiments are necessary to identify these products after chlorine treatment.

The observed degradation and chlorination reactions created numerous new arsenic species with unknown toxicity. The agreement of only one transformation product in the chlorination experiments with that in the soil experiments shows that the reaction pathways are to be governed by other factors like catalytic activities of the soils. Disinfections by chlorine or hypochlorite are standard methods for waste water, tap water treatment and cleaning of buildings for livestock. Because of their global application it can also come in contact with the feed additives roxarsone or arsanilic acid that not only the danger exist that more toxic inorganic arsenic is formed but also organic derivatives of the stock compounds.

Transformation of roxarsone by irradiation with UV and visible light. By irradiation of an aqueous solution of roxarsone with high power UV-light of a HBO 500, the organic structure of the compound was completely destroyed within some minutes to form arsenate as the major degradation product detected by HPLC-ICP-MS. Emerging organic arsenic species were not

observed during this photochemically induced reaction. To decelerate the degradation process, only light in the visible range was applied with the result that the reaction took place more slowly. However, it led also to inorganic arsenic species without detectable arsenic containing intermediates similar to the treatment with UV light. That means that photo-activated processes are not responsible for the formation of the identified and not identified transformation products. Because of the non-sterile working conditions, also biological halogenation[30,31] can take place to form the chlorinated arsenic containing compounds.

Conclusion

The treatment of roxarsone in aqueous suspensions of volcanic soils leads to numerous transformation and degradation products, which contain arsenic as heteroatom. The identification by means of elemental and molecular-specific detection can be effectively supported by complementary approaches, like ESI-MS/MS and ESI-TOF-MS.

One of the transformation products formed in contact with soils was identified as arsenic(III) containing compound, whereas the other both are chlorinated organoarsenicals. One of these compounds (3-chloro-4-hydroxy-5-nitrophenylarsonate) could be synthesized by direct chlorination of roxarsone. With intensive UV - irradiation of roxarsone in solution a fast degradation to inorganic arsenic species occurs in contrast to treatment under visible light. In the second case numerous organoarsenicals in low concentrations were formed. Future studies are in progress to identify the degradation and transformation products formed and to clarify their reaction pathways and toxicity.

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